

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
24 July 2003 (24.07.2003)

PCT

(10) International Publication Number
WO 03/059369 A1

(51) International Patent Classification⁷: **A61K 35/78**

(21) International Application Number: **PCT/KR02/01449**

(22) International Filing Date: 31 July 2002 (31.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2002/2772 17 January 2002 (17.01.2002) KR

(71) Applicants (for all designated States except US):
LIFETREE BIOTECH CO., LTD. [KR/KR]; #44-3, Omokchun-dong, Kwonsun-gu, 441-350 Suwon-si, Kyungki-do (KR). FORESTRY RESEARCH INSTITUTE [KR/KR]; #207, Cheongryangri-2-dong, Tongdaemun-gu, 130-012 Seoul (KR).

(72) Inventors; and

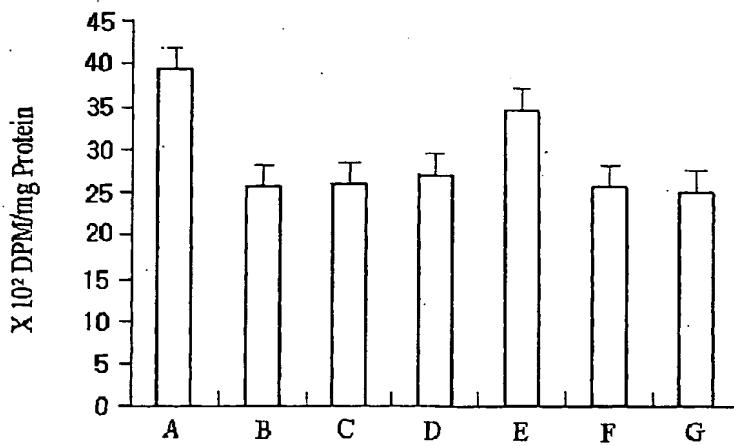
(75) Inventors/Applicants (for US only): **NA, Chun Soo [KR/KR]; Youngkwang Apt. 2-701, Hwaseo-dong, Jangan-gu, 440-150 Suwon-si, Kyungki-do (KR). JUNG, Nam Chul [KR/KR]; Dongnam Apt. 107-1102, #151, Pyung-dong, Kwonsun-gu, 441-230 Suwon-si, Kyungki-do (KR). KIM, Sea Hyun [KR/KR]; Jugong Apt. 426-304, Maetan-dong, Paldal-gu, 442-370 Suwon-si, Kyungki-do (KR).**

(74) Agents: **JANG, Seong Ku et al.; 17th Fl., KEC Building, #275-7, Yangjae-dong, Seocho-ku, 137-130 Seoul (KR).**

(81) Designated States (national): **AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI,**

[Continued on next page]

(54) Title: LOWER ALCOHOL-INSOLUBLE EXTRACT OF HOVENIA DULCIS THUNB AND A POLYSACCHARIDE ISOLATED THEREFROM



A: Control

B: Bromobenzene 1mM

C: Bromobenzene 1mM + Fr. 1 200µg/ml

D: Bromobenzene 1mM + Fr. 2 200µg/ml

E: Bromobenzene 1mM + Fr. 3 200µg/ml

F: Bromobenzene 1mM + Fr. 4 200µg/ml

G: Bromobenzene 1mM + Fr. 5 200µg/ml

(57) Abstract: A pharmaceutical composition and health care food comprising a lower alcohol-insoluble extract of *Hovenia dulcis* Thunb or a polysaccharide isolated therefrom has a potent hepatoprotective and hangover-resolving activity.

WO 03/059369 A1

BEST AVAILABLE COPY



SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN,
YU, ZA, ZM, ZW.

Published:

— *with international search report*

(84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

LOWER ALCOHOL-INSOLUBLE EXTRACT OF *HOVENIA DULCIS* THUNB AND A POLYSACCHARIDE ISOLATED THEREFROM

Field of the Invention

5

The present invention relates to a lower alcohol-insoluble extract of *Hovenia dulcis* Thunb, a polysaccharide isolated therefrom having hepatoprotective and hangover-resolving activities and a pharmaceutical composition or health care food containing same.

10

Background of the Invention

Hepatitis has been afflicting an ever-increasing number of the world population, and due to the lack of therapeutically effective drugs, it usually progresses to chronic hepatitis, liver cirrhosis or cancer. Various types of hepatitis may be developed when a patient is exposed to several factors such as stress, excessive consumption of alcohol, and/or hepatotoxic substances.

Known as exemplary hepatotoxic substances are CCl₄, D-galactosamine, lipopolysaccharide (LPS), bromobenzene and aldehydes such as acetaldehyde which is known as an intermediate in the metabolic pathway of alcohol. Accordingly, there have been many attempts to find new drugs which can protect the liver from such hepatotoxic substances or restore the liver function damaged thereby.

For example, triterpene glycosides isolated from the seed and fruit of *Hovenia dulcis* Thunb are known to inhibit the release of histamine and the absorption of alcohol in human body (Yoshikawa, K. T. et al., (1995) *Chem. Pharm. Bull. Tokyo*, **43**(3), pp532-534); and the fruit of *Hovenia dulcis* Thunb has been found to inhibit liver damage caused by carbon tetrachloride or D-galactosamine/ lipopolysaccharide (Hase K. et al., (1997) *Chem. Pharm. Bull. Tokyo*, **20**(4), pp381-385). The above and other prior art literatures describe pharmacological activities of various extracts of the fruits or seeds of *Hovenia dulcis* Thunb. var. *tomentella* Makino (Japanese species) and *Hovenia dulcis*

Thunb (Chinese species); however there have been no reports directed to the use of an alcohol-insoluble extract of *Hovenia dulcis* Thunb or a polysaccharide isolated therefrom for protecting the liver or for resolving hangover.

5 **Summary of the Invention**

Accordingly, it is an object of the present invention to provide a pharmacologically active substance extracted from *Hovenia dulcis* Thunb exhibiting hepatoprotective and hangover-resolving activities.

10 It is another object of the present invention to provide a method for isolating said substance from *Hovenia dulcis* Thunb.

It is an additional object of the present invention to provide a pharmaceutical composition for inhibiting alcohol dehydrogenase and lactic acid dehydrogenase, comprising a pharmaceutically acceptable carrier and the above-
15 described substance isolated from *Hovenia dulcis* Thunb.

It is a further object of the present invention to provide a pharmaceutical composition for preventing or inhibiting liver-related diseases and for treating hangover, comprising a pharmacologically acceptable carrier and a polysaccharide isolated from *Hovenia dulcis* Thunb.

20 It is a still further object of the present invention to provide a health care food comprising said substance and/or the polysaccharide derived from *Hovenia dulcis* Thunb.

In accordance with one aspect of the present invention, there is provided a lower alcohol-insoluble fraction obtained by treating a hot-water extract of
25 dried *Hovenia dulcis* Thunb with a lower alcohol.

In accordance with another aspect of the present invention, there is also provided a polysaccharide having a potent hepatoprotective activity, which is isolated from said lower alcohol-insoluble fraction.

30 **Brief Description of the Drawings**

The above and other objects and features of the present invention will

become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, which respectively show:

Fig. 1: a schematic procedure for preparing a methanol-insoluble fraction of an extract of the fruit-peduncle of *Hovenia dulcis* Thunb and a polysaccharide isolated therefrom;

5 Fig. 2: a DSC scan of the methanol-insoluble fraction;

Fig. 3: a MALLS spectrum of the methanol-insoluble fraction;

Fig. 4: an IR spectrum of the methanol-insoluble fraction;

10 Fig. 5: a UV spectrum of the methanol-insoluble fraction;

Fig. 6: GC spectrums of the methanol-insoluble fraction;

Fig. 7: MALLS spectrums of the methanol-insoluble polysaccharide;

Fig. 8: an IR spectrum of the methanol-insoluble fraction;

Fig. 9: an NMR spectrum of the methanol-insoluble fraction;

15 Fig. 10: in vitro protein synthesis activity of the methanol-insoluble fraction to a carbon tetrachloride-induced liver slice culture;

Fig. 11: in vitro protein synthesis activity of the methanol-insoluble fraction to a galactosamine /LPS-induced liver slice culture;

Fig. 12: in vitro protein synthesis activity of the methanol-insoluble fraction to a bromobenzene-induced liver slice culture;

20 Fig. 13: LDH release inhibitory activity of the methanol-insoluble fraction to the bromobenzene-induced liver slice culture;

Fig. 14: in vitro protein synthesis activity of the polysaccharide shown in Fig. 7 to the bromobenzene-induced liver slice culture;

Fig. 15: activity in lowering alcohol concentration in blood of the methanol-insoluble fraction and the polysaccharide shown in Fig. 7; and

25 Fig. 16: alcohol dehydrogenase activity of the methanol-insoluble fraction and the polysaccharide shown in Fig. 7.

Detailed Description of the Invention

30

The lower alcohol-insoluble fraction of a hot-water extract of *Hovenia dulcis* Thunb of the present invention may be prepared in two steps. First, a hot-

water extract of dried *Hovenia dulcis* Thunb is obtained using a high-pressure extraction procedure and then an insoluble fraction is obtained treating the hot-water extract thus obtained with a lower alcohol.

In the first step, a fruit peduncle of *Hovenia dulcis* Thunb is sliced and 5 dried in the shade. An appropriate amount of water is added to the dried slices of the *Hovenia dulcis* Thunb fruit peduncle and the resulting mixture is kept at a temperature ranging from 110 to 150 °C, preferably 120 to 125 °C, under a pressure ranging from 1 to 3 atm., preferably 1.5 atm., for a period ranging from 15 minutes to 48 hours, preferably 30 minutes to 12 hours. Then the mixture is 10 cooled to room temperature and filtered, and the filtrate is lyophilized pursuant to a conventional lyophilizing method, to obtain a hot-water extract.

The hot-water extract is further dried at room temperature, evaporated under a reduced pressure e. g., ~1.5 atm, and extracted with a lower alcohol, preferably methanol, ethanol or butanol, most preferably 100% methanol, to 15 remove lower alcohol-soluble components therefrom, to obtain the intended lower alcohol-insoluble fraction.

The above-mentioned *Hovenia dulcis* Thunb is selected from the group comprising *Hovenia dulcis* Thunb. var. *Koreana* NAKAI, *Hovenia dulcis* Thunb. var *tomentella* Makino and *Hovenia dulcis* Thunb.

20 The lower alcohol-insoluble fraction thus obtained has the following characteristics: gel permeation chromatography (GPC) peaks at mean M.W. of 1,330,000 dalton (Da), 142,800 Da, 70,540 Da and 102,400 Da; IR (KBr, nm) absorption bands at 1000-1300 nm (ether, phenol, sulfoxide, vinyl peak); and a UV absorption at 200-300 nm (cyclic ring peak).

25 A polysaccharide having high hepatoprotective and hangover-resolving activities can be isolated from the lower alcohol-insoluble fraction by the following procedure.

The lower alcohol-insoluble fraction is dissolved in distilled water, an ion exchange column is charged with the resulting solution, polysaccharide fractions 30 are eluted stepwise using solutions having increasing NaCl concentrations from 0 to 5M, dialyzed, concentrated, and lyophilized (see Fig. 1).

In carrying out the ion exchange, either a cation exchange resin or an

anion exchange resin may be used. Examples of exchange resins which can be used for this purpose are: strong acidic cation exchange resins such as AG 50W-x8, Amberlite IR-120 and Dowex 50W-x8; weak acidic cation exchange resins such as Amberlite IRC-50, Bio-Rex 70 and Duolite-436; weak basic cation exchange resins such as Amberlite IRA-67 and Dowex 3-x4A; strong basic cation exchange resins such as AG 2x8, Amberlite IRA-400 and Dowex 2-x8; modified cellulose cation exchange resins such as CM-Cellulose and SE-Cellulose; a modified cellulose anion exchange resins such as DEAE Cellulose; cationic sephadex-type resins such as G-25 and G-50 bead type cross-linked dextran resins; and modified bead-type ion exchange resins made from agarose such as Cepharose CL, Biogel A Cepharose resin, Fractogels and Toyopearl. The preferred are Toyopearl DEAE type exchange resins and the most preferred are Toyopreal DEAE-650C type exchange resins.

Several polysaccharide fractions are obtained according to the above isolation process and a polysaccharide that is eluted with 0.2 M NaCl solution shows the highest hepatoprotective activity. The polysaccharide obtained from *Hovenia dulcis* Thunb indigenous to Korea according to the above procedure shows the following characteristics: It is composed of mannose, glucose, galactose, rhamanose and arabinose in the ratio of 1: 2.51: 12.53: 187: 13.43; absolute molecular weight is 114,500 Da; and IR (KBr, nm) shows peaks at 3550-3450 Da(broad, OH), 1660-1600 Da(C=C), and 1290-1420 Da(=CH-OH); and ¹H-NMR (600MHz, D₂O) exhibits a peak at 4.4-4.8 ppm (sugar peak). Further, the polysaccharide obtained from *Hovenia dulcis* Thunb indigenous to China shows the following characteristics: It is composed of galactose, rhamanose, glucuronic acid, galacturonic acid, xylose, mannose and glucose in the ratio of 1: 0.84: 1.05: 0.87: 0.89: 2.05.

Various experiments clearly show that the lower alcohol-insoluble fraction and the polysaccharides isolated therefrom have high hepatoprotective and hangover-resolving activities and are effective for preventing and treating liver diseases and hangover.

Thus, the lower alcohol-insoluble fraction and the polysaccharide of the present invention may be employed as a health care food and a pharmaceutical

agent for preventing or treating liver toxicity and liver diseases such as hepatitis, fatty liver and liver cirrhosis.

Accordingly, the present invention also provides a pharmaceutical composition for inhibiting alcohol dehydrogenase and lactic acid dehydrogenase, 5 which comprises the lower alcohol-insoluble fraction of *Hovenia dulcis* Thunb or the polysaccharide isolated from Fraction 3 of the methanol-insoluble fractions as an active ingredient, in combination with pharmaceutically acceptable excipients, carriers or diluents.

Additionally, the present invention provides a health care food comprising 10 the extract and/or the polysaccharide described above.

Also, the present invention also provides a pharmaceutical composition for preventing and treating liver diseases and hangover, which comprises the lower alcohol-insoluble fraction of *Hovenia dulcis* Thunb and the polysaccharide isolated therefrom as an active ingredient, in combination with pharmaceutically 15 acceptable excipients, carriers or diluents. Additionally, the present invention provides a health care food comprising the extract and/or the polysaccharide described above.

The inventive pharmaceutical formulation may be prepared in accordance with any of the conventional procedures. In preparing the formulation, the active 20 ingredient is preferably admixed or diluted with a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet or other container. When the carrier serves as a diluent, it may be a solid, semi-solid or liquid material acting as a vehicle, excipient or medium for the active ingredient. Thus, the formulation may be in the form of a tablet, pill, powder, sachet, elixir, suspension, 25 emulsion, solution, syrup, aerosol, soft and hard gelatin capsule, sterile injectable solution, sterile packaged powder and the like.

Examples of suitable carriers, excipients, or diluents are lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, alginates, gelatin, calcium phosphate, calcium silicate, cellulose, methylcellulose, microcrystalline cellulose, 30 polyvinylpyrrolidone, water, methylhydroxybenzoates, propylhydroxybenzoates, talc, magnesium stearate and mineral oil. The formulation may additionally include fillers, anti-agglutinating agents, lubricating agents, wetting agents,

flavoring agents, emulsifiers, preservatives and the like. The composition of the invention may be formulated so as to provide a quick, sustained or delayed release of the active ingredient after it is administrated to a patient, by employing any one of the procedures well known in the art.

5 The pharmaceutical formulation of the present invention can be administered via various routes including oral, transdermal, subcutaneous, intravenous and intramuscular introduction. For treating a human patient, a typical daily dose of the above-mentioned fraction or polysaccharide isolated from *Hovenia dulcis* Thunb may range from about 0.01 to 10 g/kg body weight,
10 preferably 1 to 5 g/kg body weight, and can be administered in a single dose or in divided doses. However, it should be understood that the amount of the active ingredient actually administered ought to be determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the
15 severity of the patient's symptom; and, therefore, the above dose should not be intended to limit the scope of the invention in any way.

The above-mentioned lower alcohol-insoluble fraction and the polysaccharide isolated therefrom can be added to food or beverage for preventing various liver diseases and hangover. The amount of said fraction and/or
20 polysaccharide that may be added to food or beverage for the purpose of preventing liver diseases or resolving hangover may generally range from about 0.1 to 15 w/w %, preferably 1 to 10 w/w % based on the total weight of food, and 1 to 30 g, preferably 3 to 10 g based on 100 ml of beverage.

25 The health care beverage composition of the present invention may contain other components, e.g., deodorants and natural carbohydrates as in conventional beverages. Examples of such natural carbohydrates are monosaccharides such as glucose and fructose; disaccharides such as maltose and sucrose; conventional sugars such as dextrin and cyclodextrin; and sugar alcohols such as xylitol, sorbitol and erythritol. As the deodorant, a natural deodorant
30 such as taumatin, levaudioside A, and glycyrrhizin, or a synthetic deodorant such as saccharin and aspartam may be used. The amount of the above-described natural carbohydrate is generally in the range of about 1 to 20 g, preferably 5 to

12 g based on 100 ml of beverage.

Other components that may be added to the inventive food or beverage composition are various nutrients, vitamins, minerals, synthetic flavoring agents, coloring agents, pectic acid and its salt, alginic acid and its salt, organic acids, 5 protective colloidal adhesives, pH controlling agents, stabilizers, preservatives, glycerin, alcohol, carbonizing agents, fruit juices and vegetable juices.

The following Reference Examples, Test Examples and Formulation Examples are intended to further illustrate the present invention without limiting its scope.

10

Reference Example 1: Determination of absolute molecular weight by Gel Permeation Chromatography

The molecular weight measurement was made using a GPC apparatus, 15 equipped with a pump (spectra system, p2000 model), a guard column (TSK PWH, Tosoh Company), an RI-detector (Shodex SE71 model), SEC (size exclusion chromatography) columns (TSK gel 3000pw, 4000pw, 5000pw (7.8x300 mm, Tosoh Company)), and a MALLS (multi angle laser light dispersion, Dawn DSP-F, Wyatt Technology Co.) detector, using a 0.02% sodium azide as 20 developing solvent containing 0.15 M NaNO₃ at a flow rate of 0.5 ml/min.

Reference Example 2 : Reagents and materials

The amount of LDH (lactic acid dehydrogenase) was determined with a 25 340-UV spectrometer (Sigma Co., U.S.A.). Both the ³H-Leucine(5 µ Ci/plate) isotope used to determine the amount of synthesized protein having healing activity of the liver damaged by a hepatotoxic substance and the ³H-Uridine isotope used to determine the amount of synthesized RNA were purchased from Sigma Co. Absorbance variation measurement was conducted with a gas 30 chromatography head-space analytical apparatus, HP 5890 gas chromatography (Hewlett Packard Company in USA) equipped with an FID (flame ionization detector), to determine the activity of alcohol dehydrogenase.

Example 1: Preparation a lower alcohol-insoluble fraction of *Hovenia dulcis* Thunb indigenous to Korea

5 Dried and sliced peduncles (1.5 kg) of *Hovenia dulcis* Thunb indigenous to Korea was subjected to hot-water extraction at 120 °C for 3 hours under a high pressure (1.5 atm). The resulting extract solution was filtered through Wattman paper. The filtrates of *Hovenia dulcis* Thunb indigenous to Korea (218.5 g) was lyophilized, the resulting powder was subjected to 3 cycles of reflux-extraction,
10 each with 3 ℥ of HPLC-grade pure methanol for 1 hour and the remaining powder was dried to obtain a methanol-insoluble fraction of *Hovenia dulcis* Thunb indigenous to Korea (dry weight 65.71 g, yield: 4.3% w/w). Also, the extracts were combined and lyophilized to obtain a methanol-soluble fraction (dry weight 152.29 g, yield: 10.27% w/w).

15

Example 2: Analysis of the methanol-insoluble fraction of *Hovenia dulcis* Thunb

The properties of the methanol-insoluble fractions obtained in Example 1 were analyzed as follows.

20

(1) Determination of the melting temperature and the melting enthalphy

The melting temperature and the melting enthalphy were determined by DSC (Differential Scanning Calorimeter, Seiko Instruments Inc. DSC 6100).
25 Samples of the methanol-insoluble fraction of *Hovenia dulcis* Thunb indigenous to Korea was placed in an aluminum pan, sealed, and then heated from 20 °C to 200 °C at a rate of 10 °C/min to obtain a melting heat absorption curve and the melting temperature, and the crystallinity of the sample was determined therefrom, respectively.

30 The DSC scans of the methanol-insoluble fractions indigenous to Korea showed that a main peak starting at 164.9 °C and reaching a maximum melting temperature 185.3 °C. The methanol-insoluble fraction was formed to several

10

carbohydrates (mp: 60~100 °C) and a minor amount of proteins (mp: 60~100 °C) (Fig. 2).

5 (2) Analysis for sugar chains

To examine whether the above main-peak components contain sugar chains, a methylation analysis was conducted according to the method described by Hakomori et al. (*J. Biochem. Tokyo*, 55, 205-209, 1964) and Waeghe T.J. et al. (*Carbohydrate Research*, 123, 281-304, 1983).

10 A 500 µg sample was methylated, and then, the methylated product was collected using an ethanol-adsorbed C₁₈ 8x10 cartridge column (Sep-Pak). Acidic sugar moieties of the methylated product were reduced using LiB(C₂H₅)₃D (Super-Deupride, 1 ml, Aldrich Company) in THF and the reduction product was recovered using a C₁₈ 8x10 cartridge column(Sep-Pak). Subsequently, the treated sample was subjected sequentially to: hydrolysis at 121 °C for 2 hours in 1.0 M TFA; reduction by NaBD₄; and acetylation. The resulting partially methylated alditol acetate was analyzed by GLC and GC-EIMS, and the peak areas were measured with an FID (flame ionization detector).

15 20 (3) Determination of molecular weight by GPC

The result of GPC conducted as in Reference Example 1 showed that the methanol-insoluble fraction was composed of 4 peak components as shown in Fig. 3 and in Table 1.

25 Table 1. Peak components of methanol-insoluble fraction

Components	Peak 1	Peak 2	Peak 3	Peak 4
Mean M.W. (Da)	1,330,000	142,800	70,540	102,400

(4) IR spectrum (Fig. 4)

The sample was analyzed by IR spectroscopy (Vector 22 model, Bruker Analytische Messtechnik GMBH): resolution, 4.0; source, sphere; velocity, 6, 10 KHz; capture mode, dual wall/forward-backward condition).

5 IR (KBr, cm⁻¹): ether, phenol, sulphoxide and vinyl peak (1000-1300nm; main 1039nm), aromatic ring peak (665, 939, 1313, 1663), hydroxyl peak (3435nm).

(5) UV spectrum (Fig. 5)

10 The sample was analyzed by UV-Vis spectroscopy (HP 8453 model, Hewlett Packard Company) and the result showed the maximum absorbance at 200-300 nm, suggesting the existence of a cyclic ring.

15 Example 3: Isolation of polysaccharides having hepatoprotective and hangover-resolving activities

To isolate active compounds having hepatoprotective and hangover-resolving activities from the methanol-insoluble fraction obtained in Example 1,
20 200 mg of the methanol-insoluble fraction was dissolved in distilled water, charged to a Toyopearl® DEAE-650C column (4.0x 30 cm), and eluted successively with 0, 0.1, 0.2, 0.3 and 3M NaCl solutions. The eluted fractions were dialyzed using a dialysis membrane permeable at a M.W. of 1000 or below, concentrated, and then lyophilized to obtain purified fractions weighing 38 mg, 64
25 mg, 73 mg, 5 mg and 4 mg, respectively.

Example 4: Characterization of the isolated fractions of *Hovenia dulcis* Thunb indigenous to Korea

30 The isolated fractions obtained in Example 3 by eluting with 0, 0.1, 0.2, 0.3 and 3 M NaCl were designated as Fractions 1 to 5, respectively. For each fraction, the contents of total sugar and polyphenol components were determined

by the phenol-sulfuric acid method (Dubois M. et al.; *Anal. Chem.* 28, 350-356, 1956), and the result thus obtained is shown in Table 2.

Table 2. Contents of total sugar and polyphenol components

5

Constituent	Content (ppm)				
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Total sugar	248.79	231.21	122.76	51.81	2.33
Polyphenol	0	0.0245	0	0	0

GC analyses were conducted (Varian CP-3800 model, set-up condition; detector : FID, column: SP-2380 (30 m x 0.25 mm x 0.2 μm), temperature of column: 230 °C, temperature of injector: 250°C, temperature of detector: 250°C, mobile phase: N₂ gas(1.0ml/min)) to identify the sugar components of the above fractions and the relative amounts of mannose, glucose, galactose, ramanose, arabinose and xylose present in each fraction were determined . The result is shown in Table 3.

15 Table 3. Amounts of sugar components (relative to mannose)

	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Mannose	1	1	1	1	1
Glucose	6	3.29	2.51	1.13	3.05
Galactose	3.1	2.04	12.53	7.81	-
Ramanose	-	0.32	187	3.64	-
Arabinose	1.58	1.93	13.43	5.86	1.93
Xylose	-	0.26	-	0.75	-

Fraction 3 showed the highest hepatoprotective and hangover-resolving activities among the above fractions, and it was further characterized as below.

20

Characterization of Fraction 3

The absolute molecular weight of Fraction 3 determined by the procedure

of Reference Example 1 was 114,500 Da (Fig. 7). An IR analysis (KBr) showed peaks (cm^{-1}) at 3550-3450 (broad, OH), 1660-1600 (C=C) and 1290-1420 (=CH-OH) (Fig. 8). $^1\text{H-NMR}$ (600MHz, D_2O) showed a peak at 4.4-4.8ppm (sugar peak) (Fig. 9).

5

Example 5: Characterization of the isolated fractions of *Hovenia dulcis* Thunb indigenous to China

The procedure of Example 1 was repeated to obtain a methanol-insoluble fraction (dry weight 61.5 g, yield: 4.1% w/w) and a methanol-soluble fraction (dry weight 133.5 g, yield: 8.9% w/w) from the fruit-peduncles of *Hovenia dulcis* Thunb indigenous to China. The methanol-insoluble fraction was subjected to ion-exchange chromatography according to the method of Example 3 and the isolated fractions obtained by eluting with 0, 0.1, 0.2, 0.3 and 3 M NaCl were designated as Fractions 1' to 5', respectively.

Fractions 1' to 5' were characterized according to the procedure of Example 4. The sugar components were identified by GC analyses and the result is shown in Table 4.

20 Table 4. Amounts of sugar components (relative to galactose)

	Fraction 1'	Fraction 2'	Fraction 3'	Fraction 4'	Fraction 5'
Galactose	1.00	1.00	1.00	1.00	1.00
Ramanose	0.58	0.57	0.84	0.77	-
Glucuronic acid	0.87	0.78	1.05	1.47	0.87
Galacturonic acid	0.88	0.30	0.87	0.97	0.98
Xylose	0.55	-	-	-	-
Mannose	0.91	-	0.89	-	1.09
Glucose	1.50	0.41	2.05	4.00	1.88

The SEC-RI peaks and absolute molecular weights were determined according to the procedure of Reference Example 1. The results are shown in

Tables 5 and 6, respectively.

Table 5. Peak component ratio of methanol-insoluble fraction by SEC-RI detector

	Fraction 1'	Fraction 2'	Fraction 3'	Fraction 4'	Fraction 5'
Peak 1	13%	35%	5%	2%	3%
Peak 2	64.6%	60%	89%	92%	89%
Peak 3	15%	1%	5%	4%	4%
Peak 4	7%	3%	-	2%	4%

5

Table 6. Measurement of the absolute molecular weight methanol-insoluble fractions by SEC-MALLS

	Fraction 1'	Fraction 2'	Fraction 3'	Fraction 4'	Fraction 5'
Peak 1	151,500	37,750	4,132,000	573,500	258,900
Peak 2	87,930	74,770	516,500	518,900	35,630
Peak 3	ND	ND	23,350	ND	ND
Peak 4	ND	ND	-	ND	ND
ND : not determined					

10 Test Example 1: Hepatoprotective activity of the methanol-insoluble fraction of *Hovenia dulcis* Thunb

(1) Protective activity for the liver damaged by carbon tetrachloride

Livers taken out from five-week old Sprague-Dawley rats were sliced into disc-shaped samples each having a diameter of about 0.8 mm and a thickness of 200 μm (wet weight: 18-22 mg), using a tissue cutter (Brendel/Vitron Co., USA). The sliced samples were divided into 4 groups, two(group 3 and group 4) of which were treated with the methanol-insoluble fraction and the methanol-soluble fraction prepared in Example 1, respectively, each at a concentration of 200 $\mu\text{g}/\text{mL}$. Then, 4 sliced samples were surface-cultured under an atmosphere of $\text{O}_2/\text{CO}_2=95\%/5\%$ in a thermodynamic organ tissue cultivator (Sankyo Co., Japan). After 1 hour, carbon tetrachloride was added to a concentration of 4mM to each of group 3, group 4 and one of the remaining two groups (group 2). The other group (group

1) was treated with distilled water instead of carbon tetrachloride (control). And then all groups were kept for 5 hours to induce liver damages.

Thereafter, the liver detoxification efficacy of the extract fraction of *Hovenia dulcis* Thunb was evaluated by determining the amount of synthesized protein according to the method described by Bonney et al. ("Some Characteristics and Function of Adult Rat Liver Primary Culture, in Gene Expression and Carcinogenesis in Cultured Liver", (1975) Gershenson, E and Thompson, E. B. (Eds), Academic Press, New York, pp24-45). As shown in Figure 10a, it was demonstrated that the methanol-insoluble fraction has a much higher activity than the methanol-soluble fraction.

Further, the result of Fig. 10b was obtained by using the methanol-insoluble fraction or methanol-soluble fraction prepared in Example 5 according to the above method, wherein the methanol-insoluble fraction shows a much higher than the methanol-soluble fraction.

15

(2) The activity for restoring the liver function damaged by D-galactosamine/LPS

It is known that administration of D-galactosamine, together with bacterial lipopolysaccharide(LPS), causes liver damage which is biochemically and histologically similar to that caused by human hepatitis. The detoxification effect of the methanol-insoluble fraction of *Hovenia dulcis* Thunb on such damaged liver was examined as follows.

The procedure of Test Example 1 was repeated using 500 μ M of D-galactosamine and 1 μ g/ml of LPS in place of carbon tetrachloride to measure the activity for restoring a damaged liver. As shown in Figure 11a, both of the methanol-insoluble and methanol-soluble fractions had significant restoration activities. Further, the result of Fig. 11b shows that the methanol-insoluble and methanol-soluble fractions obtained in Example 5 also exhibit significant restoration activities.

30

(3) Restoring the liver damaged by bromobenzene

Except for using 1 mM of bromobenzene instead of carbon tetrachloride, the detoxification efficacy of the extract of *Hovenia dulcis* Thunb indigenous to Korea or China was evaluated by using the procedure described in (1). The results, shown in Figures 12a (Korea) and 12b (China), suggest that the methanol-insoluble fractions are more potent than the methanol-soluble fraction in restoring the damaged liver.

Additionally, the amount of LDH (lactic acid dehydrogenase) released from the culture medium was determined by using a Sigma kit 340-UV apparatus and the results, Figures 13a (Korea) and 13b (China), show that the methanol-insoluble fraction is more potent than the methanol-soluble fraction in inducing the release of LDH caused by bromobenzene.

Test Example 2: Hepatoprotective activity of the polysaccharide isolated from the methanol-insoluble fraction of *Hovenia dulcis* Thunb

15

Livers taken out from five-week old Sprague-Dawley rats were sliced using a tissue cutter (Brendel/Vitron Co., USA) to obtain disc shaped samples each having a diameter of about 0.8 mm and a thickness of 200 μm (wet weight: 18-22 mg). The sliced samples were divided into 7 groups, five of which were treated with the five polysaccharide fractions prepared in Example 4, respectively, each at a concentration of 200 $\mu\text{g}/\text{ml}$ (groups 1 to 5, respectively). Then, the sliced samples were surface-cultured for 1 hour under an atmosphere of $\text{O}_2/\text{CO}_2=95\% / 5\%$ in a thermodynamic organ tissue cultivator (Sankyo Co., Japan). Then, bromobenzene was added to a concentration of 4 mM to each sample of groups 1 to 5 as well as to one of the remaining two groups (group 6). The last remaining group (group 7) was treated with distilled water in place of bromobenzene (control).

Thereafter, the liver detoxification efficacy of each polysaccharide fraction of *Hovenia dulcis* Thunb indigenous to Korea was evaluated by determining the amount of synthesized protein according to the method described by Bonney et al. (*supra*). The result, shown in Figure 14, demonstrates that the polysaccharide of Fraction 3 has the highest activity.

Test Example 3: Hangover-resolving activity of the polysaccharide isolated from the methanol-insoluble fraction of *Hovenia dulcis* Thunb

5 To measure hangover-resolving activity of the methanol-insoluble fraction of *Hovenia dulcis* Thunb obtained in Example 1, fifteen three-week old Sprague-Dawley rats were divided into 3 groups and put on a 24 hour-fast while allowing water. Then 2 ml of 40% ethanol was administered orally to each rat using a stainless-steel Sonde (length: 10 cm). After 1 hour, 2 ml of a 500 mg/ml solution
10 of the methanol-insoluble fraction or the polysaccharide (Fraction 3) isolated therefrom was administered to each rat of the experimental groups(groups 2 and 3), and the rats of the control(group 1), with 2 ml of water. After 4 hours, the alcohol concentration in a blood sample taken from the heart of each rat was measured according to the method described by Bergmeyer (In Methods of
15 Enzymatic Analysis, 3rd Ed., 598-6062, 1984).

The result (Fig. 15) showed that the alcohol concentration in blood samples taken out from the groups 1 to 3 are 0.058%, 0.032% and 0.028%, respectively. Namely, the methanol-insoluble fraction of *Hovenia dulcis* Thunb and the polysaccharide isolated therefrom lower the blood alcohol concentration
20 by 44.8% and 51.7%, respectively, as compared with the control group. This result demonstrates that the alcohol-insoluble fraction of *Hovenia dulcis* Thunb and the polysaccharide isolated therefrom have significant activities in lowering the alcohol concentration in blood.

Alcohol dehydrogenase activity in damaged liver was examined as
25 follows.

The rats were suffocated by CO₂ and livers were taken out from the rats and washed with distilled water. Each liver sample was put in 10-fold volume of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.154 M KCl and homogenized with Teflon-glass homogenizer. The homogenized solution was
30 subjected to centrifugation at 9,000 x g for 30 minutes at 4 °C and the resulting supernatant was subjected to ultra-centrifugation at 110,000 x g for 1 hour at 4 °C to obtain a supernatant cytosol fraction. 2-3 mg of the cytosole fraction was

added to a reaction mixture comprising 55 mM sodium pyrophosphate buffer(pH 7.4), 20 mM ethanol and 0.2 mM NAD. The concentration of NAD used in the reaction mixture was varied within the range of 0.025 mM to 2 mM. The change of absorbance of the reaction mixture at 340 nm was measured for 3 minutes and
5 the alcohol dehydrogenase activity was calculated from the slope of the absorbance curve.

As shown in Figure 16, the methanol-insoluble fraction and the polysaccharide isolated from Fraction 3 of the methanol-insoluble fractions showed increased alcohol dehydrogenase activity, thereby demonstrating their
10 superior hangover-resolving activity.

The lower alcohol-insoluble fraction of the present invention and the polysaccharide isolated therefrom can be used in preparing a pharmaceutically effective powder, tablet, capsule, injection or liquid composition according to any
15 one of the known conventional methods, as exemplified below.

[Formulation Example 1]

2 g of the dried extract obtained in Example 1 was mixed with 1 g of lactose to obtain a powder preparation, which was filled and sealed in a sealed
20 package.

[Formulation Example 2]

100 mg of the dried extract obtained in Example 1, 100 mg of corn starch, 100 mg of lactose, and 2 mg of magnesium stearate were mixed and tabletted to
25 obtain a tablet preparation.

[Formulation Example 3]

100 mg of the dried extract obtained in Example 1, 100 mg of the corn starch, 100 mg of lactose; 2 mg of magnesium stearate were mixed and filled in a
30 gelatin capsule to obtain a capsule preparation.

[Formulation Example 4]

100 mg of the dried extract obtained in Example 5, distilled water, and an appropriate amount of a pH controller were dissolved to obtain an injection formulation, which was filled in a 2 ml ample and sterilized according to a conventional injection preparation method, to obtain a injection preparation.

5

The health care food was exemplarily prepared by the following method.

[Preparation of health care food]

A scorched dried meal mixture of brown rice, barley, glutinous rice and
10 Job's tear was pulverized and sieved to obtain grain particles of 60 mesh or less. Also, a mixture of black bean, black sesame and wild sesame was steamed, dried, scorched, pulverized and sieved to obtain seed particles of 60 mesh or less.

The dried methanol-insoluble fraction of *Hovenia dulcis* Thunb obtained in Example 1 was pulverized and sieved to obtain particles of 60 mesh or less,
15 which were mixed with the grain particles and seed particles in the following proportions to prepare a granule type health food.

Grains : brown rice 30 w%, Job's tear 15 w%, barley 20 w%,
Seeds : wild sesame 7 w%, black bean 8 w%, black sesame 7 w%,
20 Dried powder of *Hovenia dulcis* Thunb. var. *Koreana* NAKAI : 3 w%,
Shiitake mushroom 0.5 w%, rehmania root 0.5 w%

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and
25 changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

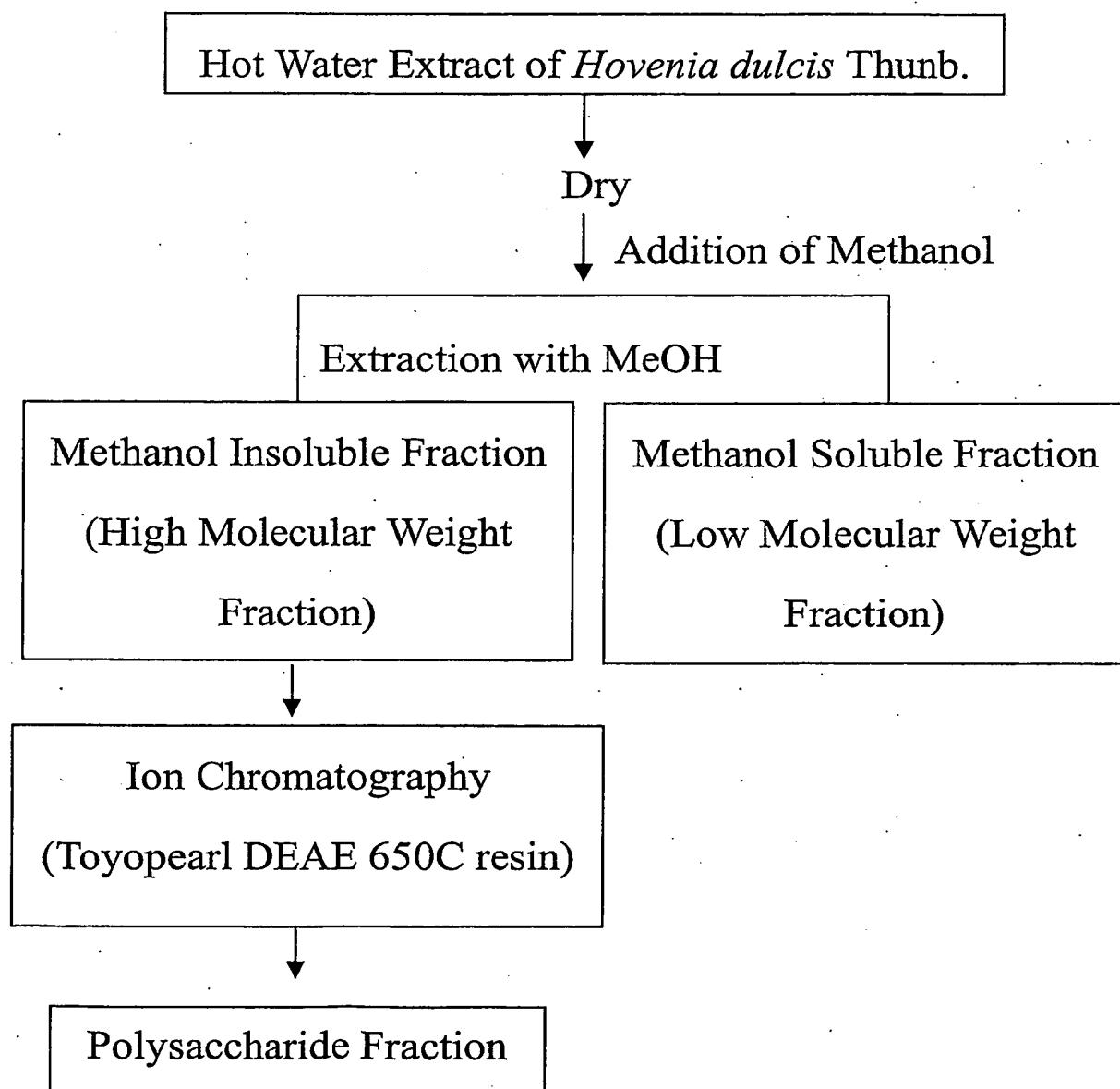
What is claimed is :

1. A lower alcohol-insoluble fraction of a hot-water extract of *Hovenia dulcis* Thunb.
- 5 2. The lower alcohol-insoluble fraction of claim 1, wherein the lower alcohol is selected from the group consisting of methanol, ethanol and butanol.
3. The lower alcohol-insoluble fraction of claim 2, wherein the lower alcohol is methanol.
- 10 4. A polysaccharide which is isolated from the lower alcohol-insoluble fraction of claims 1 to 3.
5. The polysaccharide of claim 4 which has the characteristics of: composition of mannose, glucose, galactose, rhamanose and arabinose in the ratio of 1: 2.51: 12.53: 187: 13.43; absolute molecular weight: 114,500 Da; and IR (KBr, nm) peaks: 3550-3450 Da (broad, OH), 1660-1600 Da (C=C) and 1290-1420 Da (=CH-OH); and ¹H-NMR (600MHz, D₂O) peak: 4.4-4.8 ppm (sugar peak).
- 20 6. The polysaccharide of claim 4 which has the characteristics of: composition of galactose, rhamanose, glucuronic acid, galacturonic acid, xylose, mannose and glucose in the ratio of 1: 0.84: 1.05: 0.87: 0.89: 2.05.
7. A process for preparing the lower alcohol-insoluble fraction of any one of claims 1 to 3, comprising the steps of: obtaining a hot-water extract of dried *Hovenia dulcis* Thunb; and subjecting the hot water extract to a lower alcohol extraction treatment to obtain the lower alcohol-insoluble fraction.
- 25 8. A process for preparing the polysaccharide of claim 4, comprising the steps of: obtaining a hot-water extract of dried *Hovenia dulcis* Thunb; treating the hot-water extract with a lower alcohol to obtain the lower alcohol-insoluble fraction; and subjecting the alcohol-insoluble fraction to an ion exchange column chromatography to obtain the polysaccharide.
- 30 9. The process of claim 7 or 8, wherein the lower alcohol is selected from the group consisting of methanol, ethanol and butanol.

10. The process of claim 9, wherein the lower alcohol is methanol.
11. The process of claim 8, wherein the ion exchange column chromatography is conducted using a cation exchange resin or an anion exchange resin.
- 5 12. The process of claim 11, wherein the ion exchange column chromatography is conducted using a modified bead-type ion exchange resin selected from the group consisting of Cepharose CL, Biogel A Cepharose resin, Fractogels and Toyopearl resin.
13. The process of claim 12, wherein the ion exchange column chromatography is conducted using the Toyopearl resin.
- 10 14. A composition for resolving hangover, comprising the lower alcohol-insoluble fraction of claim 1 or the polysaccharide of claim 4, and a pharmaceutically acceptable carrier.
15. A pharmaceutical composition for preventing or treating a liver disease, comprising the lower alcohol-insoluble fraction of claim 1 or the polysaccharide of claim 4, and a pharmaceutically acceptable carrier.
16. The pharmaceutical composition of claim 15, wherein the liver disease is hepatitis or liver cirrhosis.
17. A health care food comprising the lower alcohol-insoluble fraction of claims 1 or the polysaccharide of claim 4, and a sitologically acceptable additive.
- 20 18. The health care food of claim 17, wherein the food is of a beverage type.

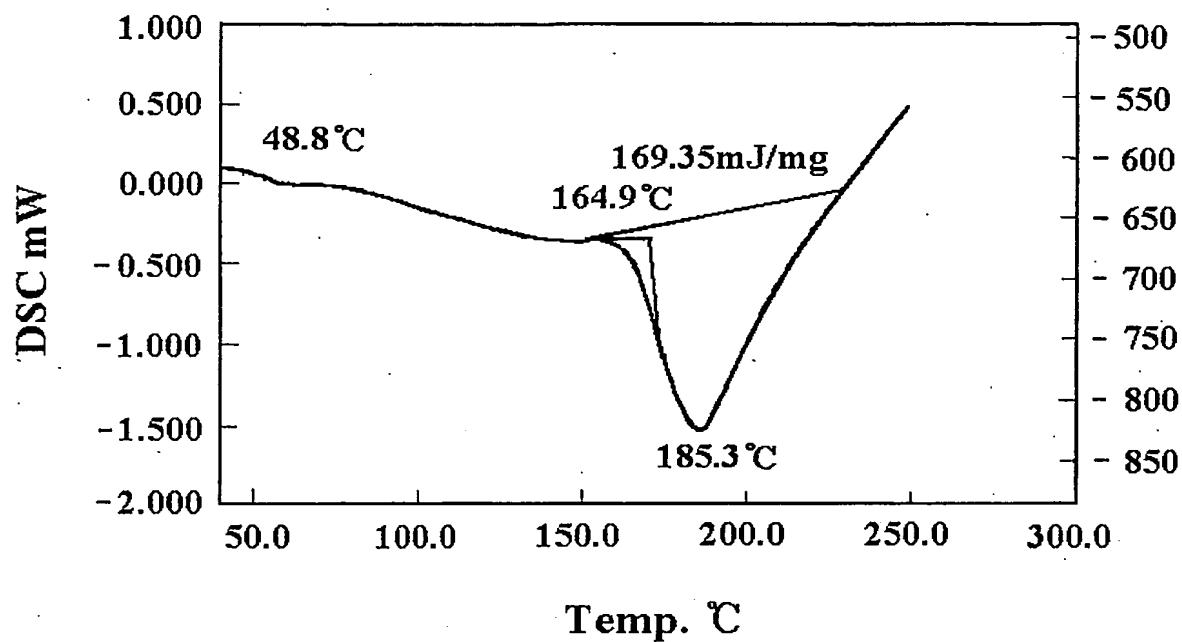
1/20

FIG. 1



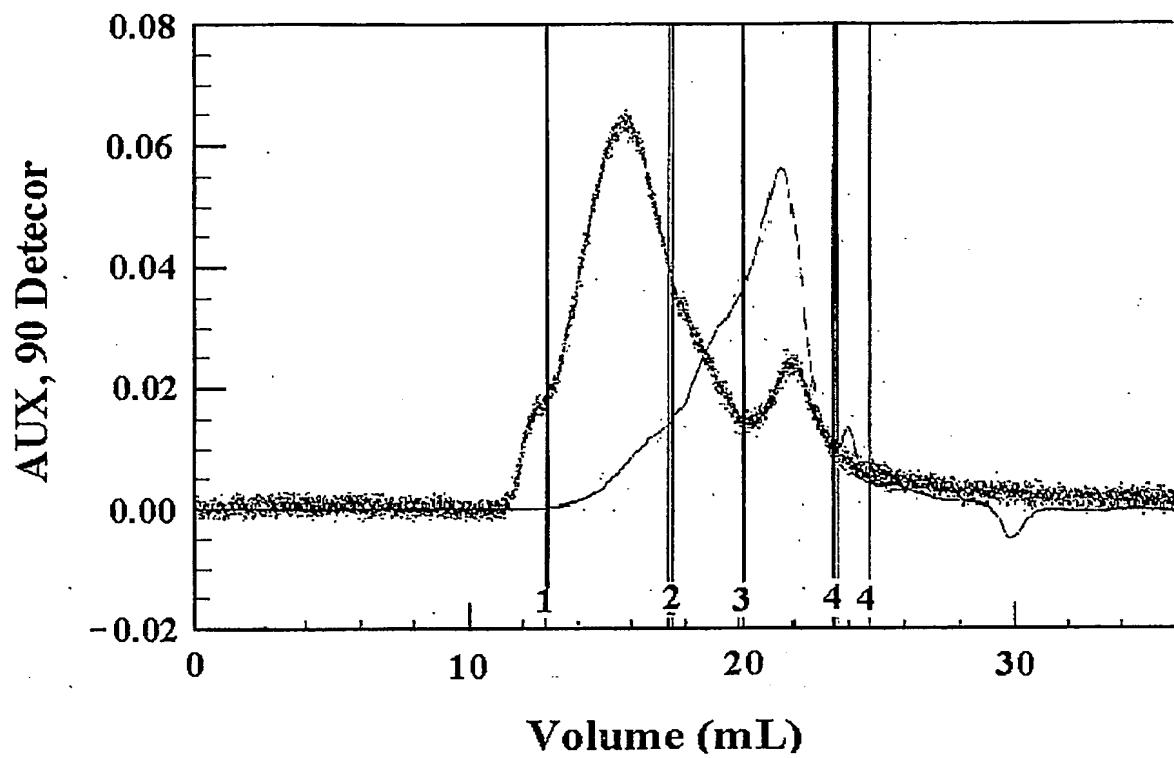
2/20

FIG. 2



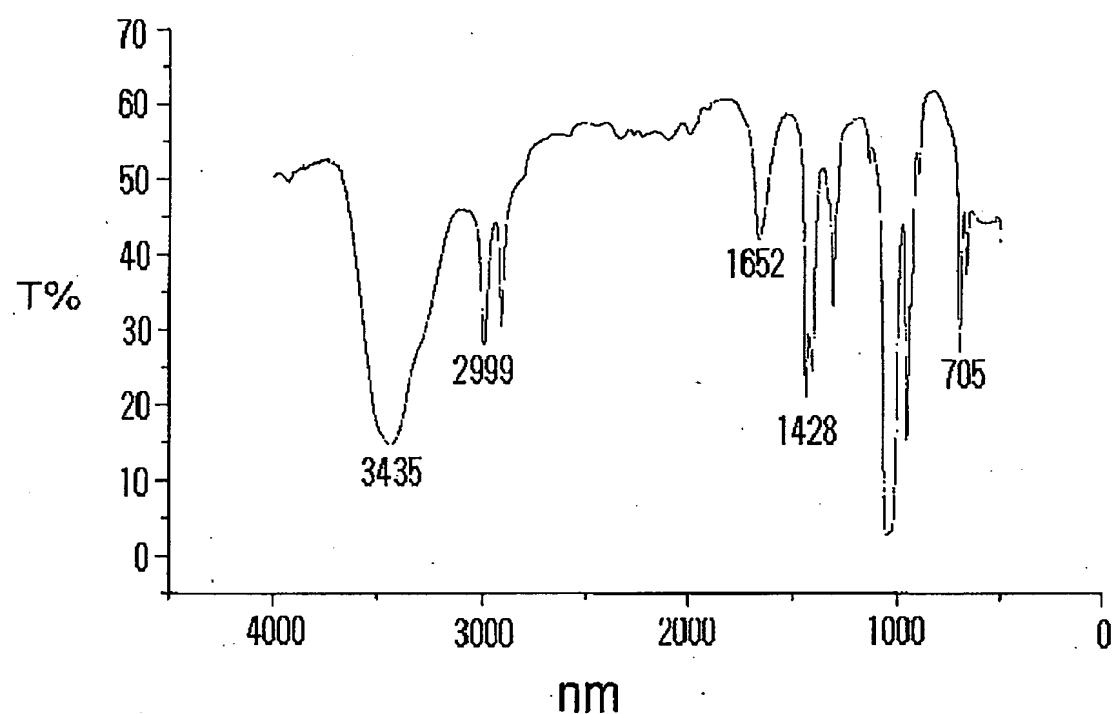
3/20

FIG. 3



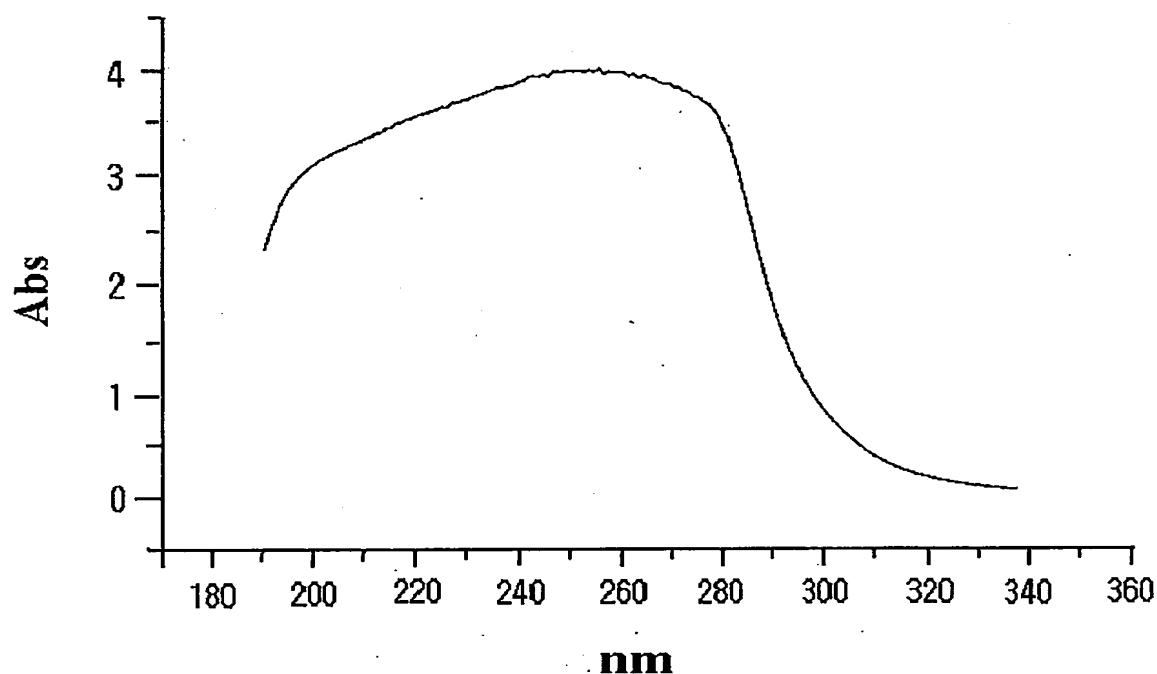
4/20

FIG. 4



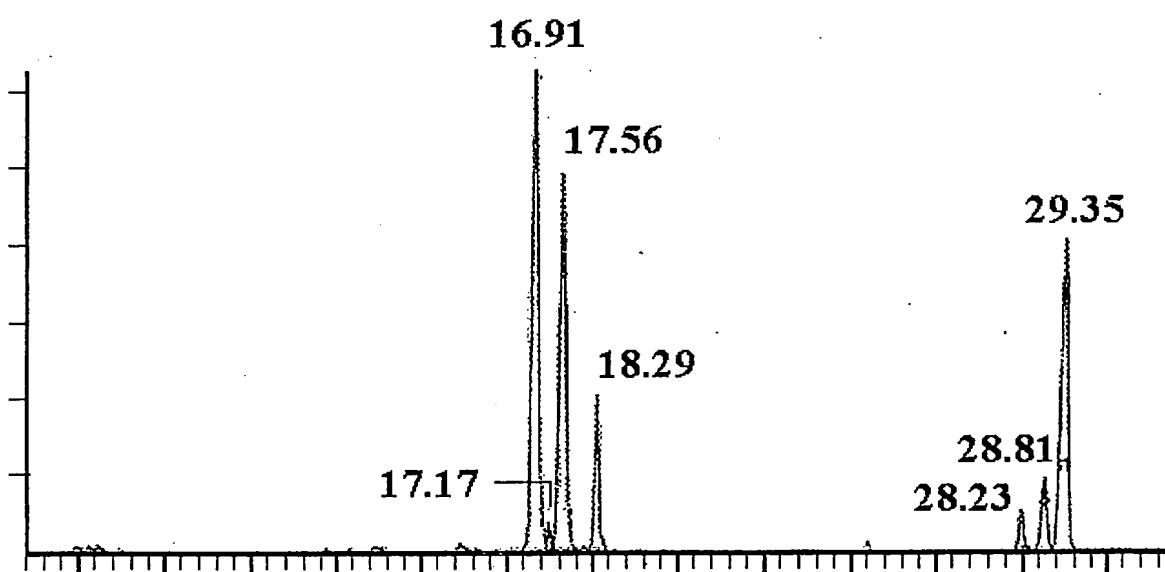
5/20

FIG. 5



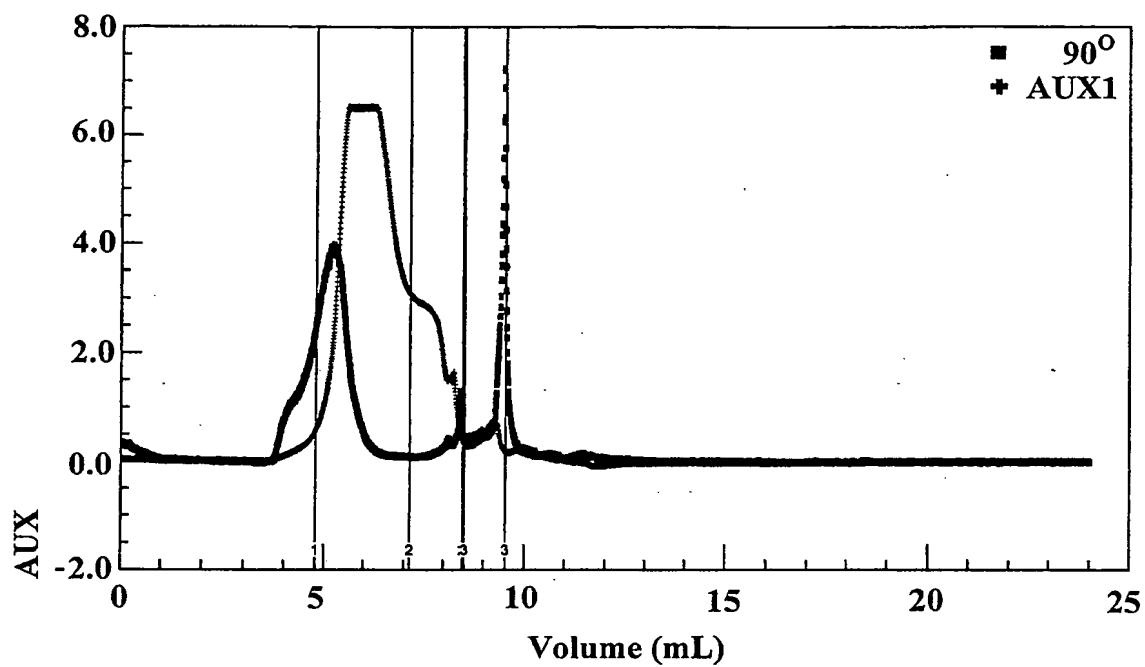
6/20

FIG. 6



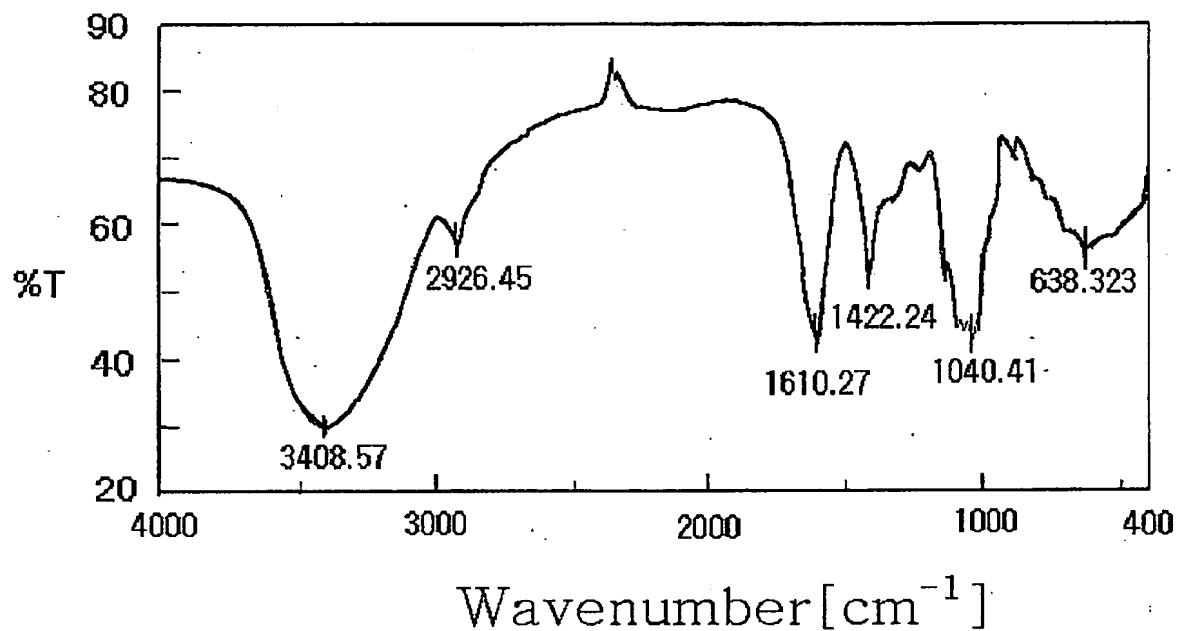
7/20

FIG. 7



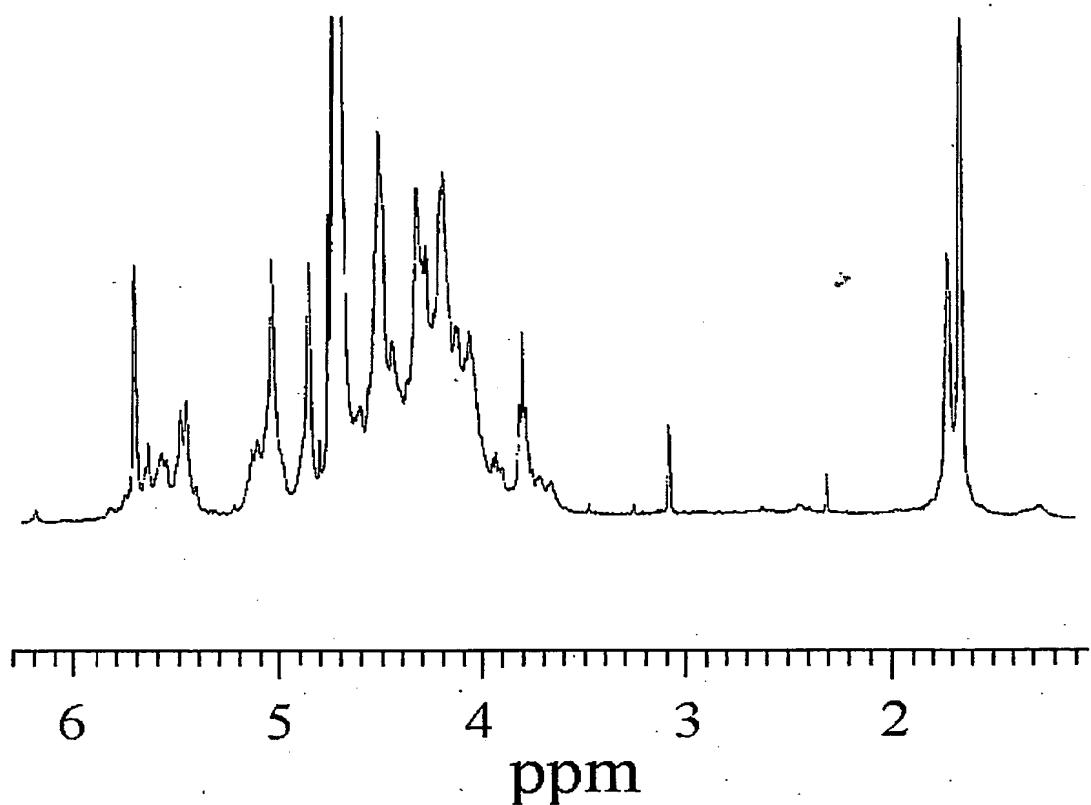
8/20

FIG. 8



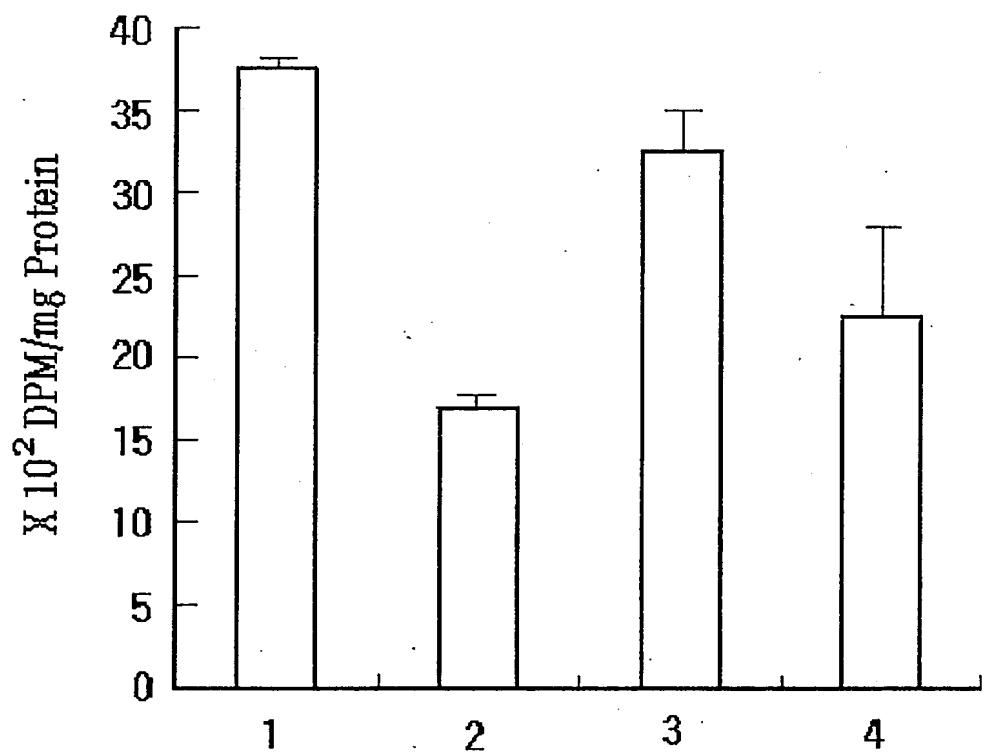
9/20

FIG. 9



10/20

FIG. 10a

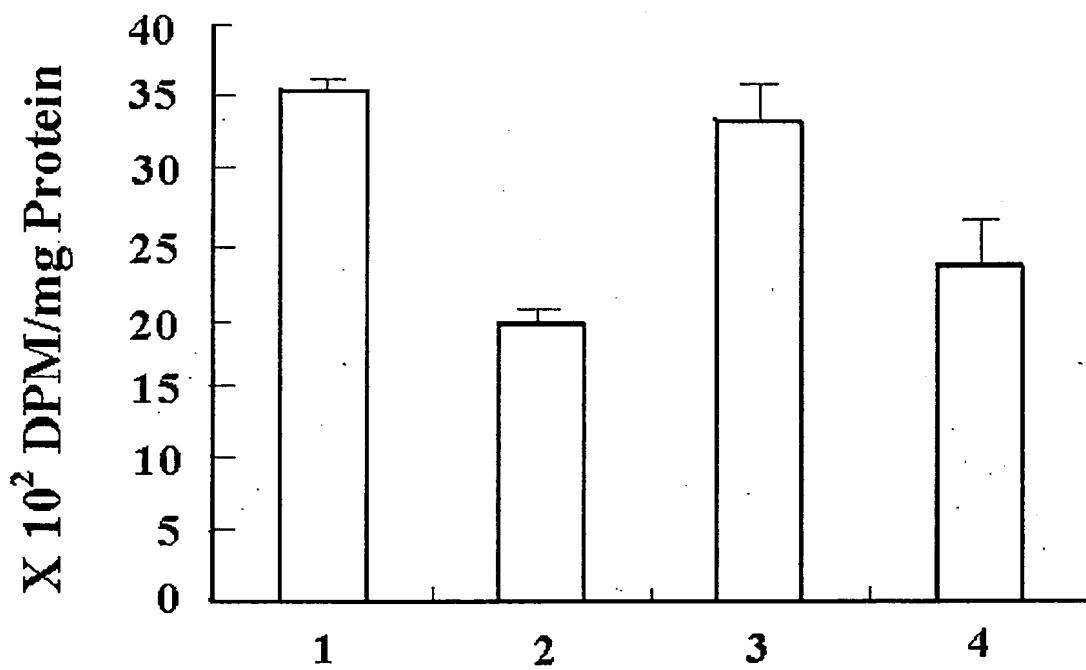


1 : Control

2 : CCl₄ 4mM3 : CCl₄ 4mM + MeOH Insoluble Fr. 200µg/ml4 : CCl₄ 4mM + MeOH Soluble Fr. 200µg/ml

11/20

FIG. 10b

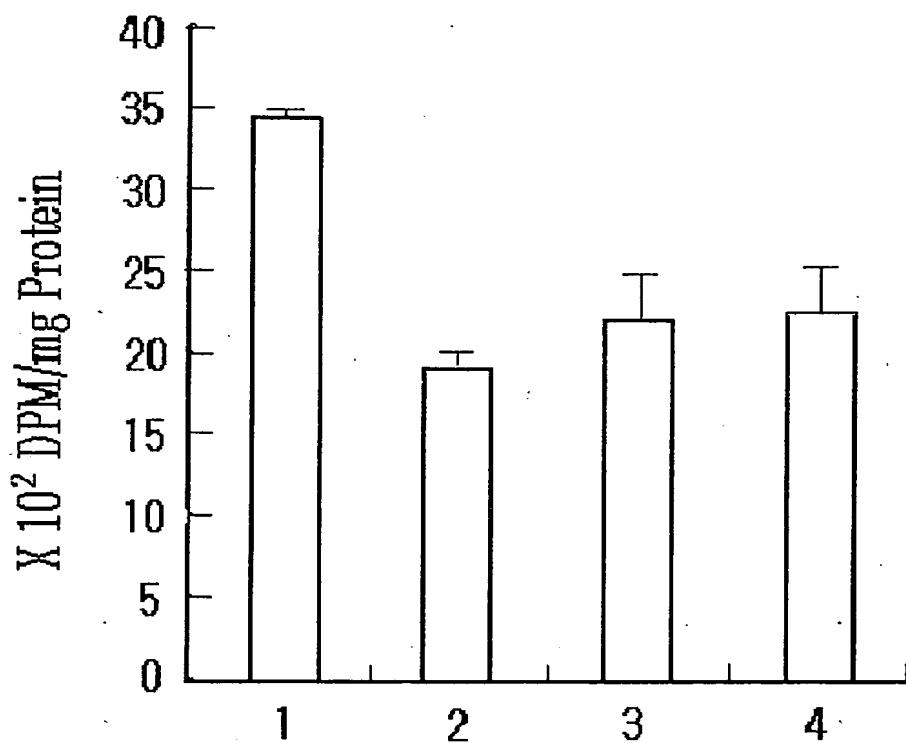


1 : Control

2 : CCl_4 4mM3 : CCl_4 4mM + MeOH Insoluble Fr. 200 $\mu\text{g/ml}$ 4 : CCl_4 4mM + MeOH Soluble Fr. 200 $\mu\text{g/ml}$

12/20

FIG. 11a

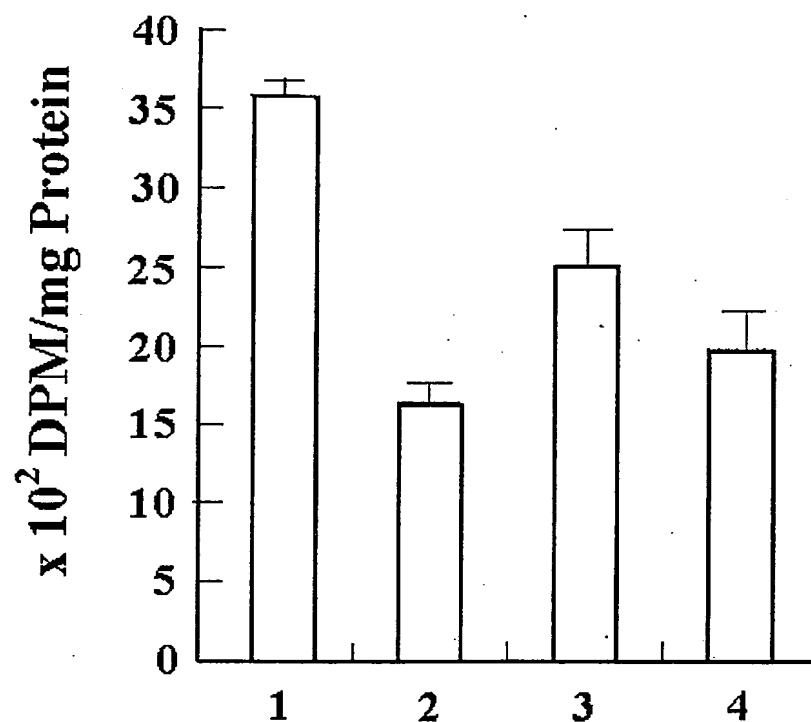


1 : Control

2 : D-Galactosamine 500 μ M + LPS 1 μ g/ml3 : D-Galactosamine 500 μ M + LPS 1 μ g/ml+ Methanol Insoluble Fr. 200 μ g/ml4 : D-Galactosamine 500 μ M + LPS 1 μ g/ml+ Methanol Soluble Fr. 200 μ g/ml

13/20

Fig. 11b

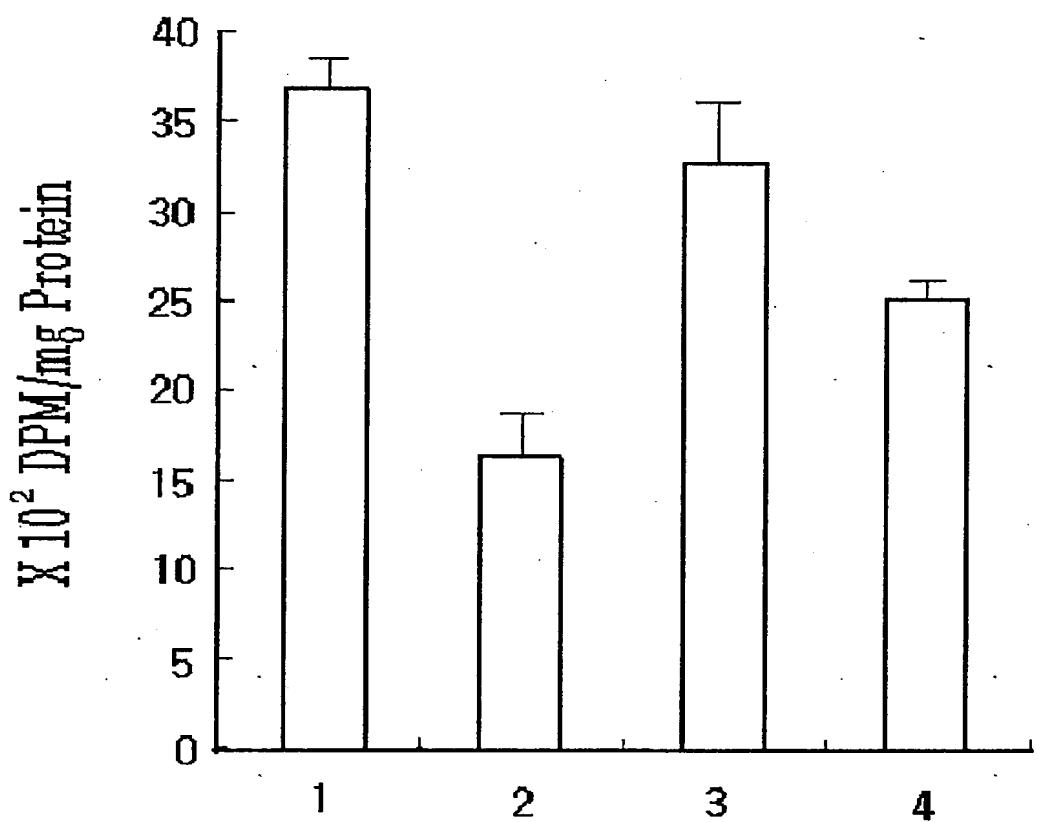


1 : Control

2 : D-Galactosamine 500 μ M + LPS 1 μ g/ml3 : D-Galactosamine 500 μ M + LPS 1 μ g/ml+ Methanol Insoluble Fr. 200 μ g/ml4 : D-Galactosamine 500 μ M + LPS 1 μ g/ml+ Methanol Soluble Fr. 200 μ g/ml

14/20

FIG. 12a



1: Control

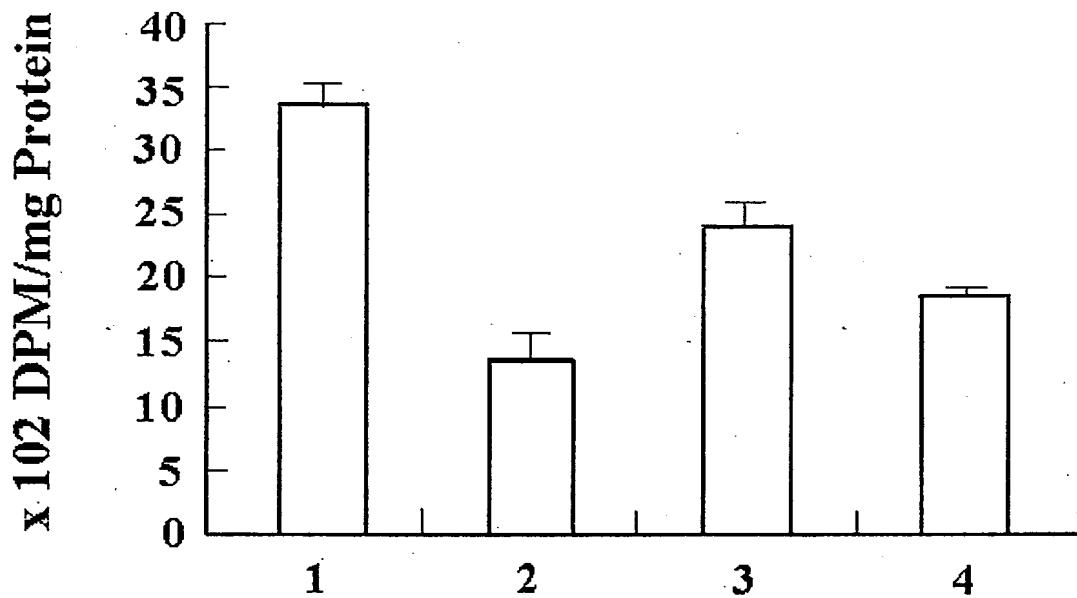
2: Bromobenzene 1mM

3: Bromobenzene 1mM + Methanol Insoluble Fr. 200µg/ml

4: Bromobenzene 1mM + Methanol Soluble Fr. 200µg/ml

15/20

Fig. 12b



1: Control

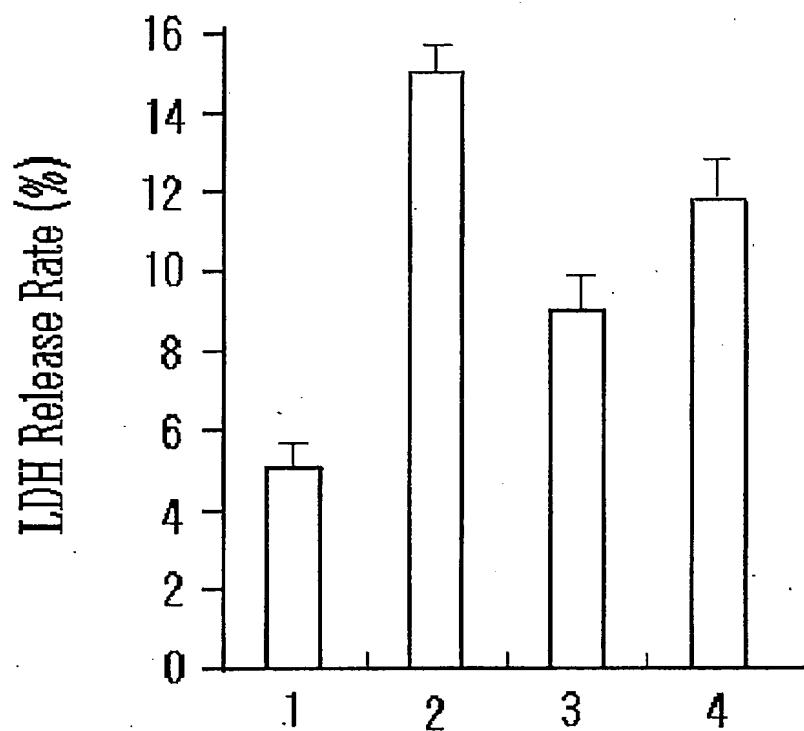
2: Bromobenzene 1mM

3: Bromobenzene 1mM + Methanol Insoluble Fr. 200µg/ml

4: Bromobenzene 1mM + Methanol Soluble Fr. 200µg/ml

16/20

FIG. 13a



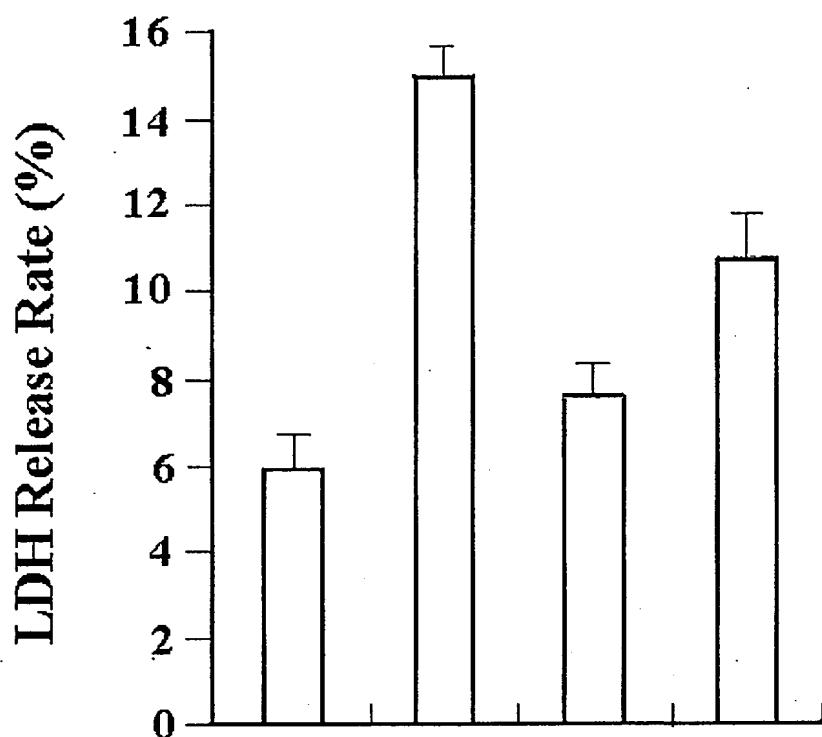
1: Control

2: Bromobenzene 1mM

3: Bromobenzene 1mM + Methanol Insoluble Fr. 200 μ g/ml4: Bromobenzene 1mM + Methanol Soluble Fr. 200 μ g/ml

17/20

Fig. 13b



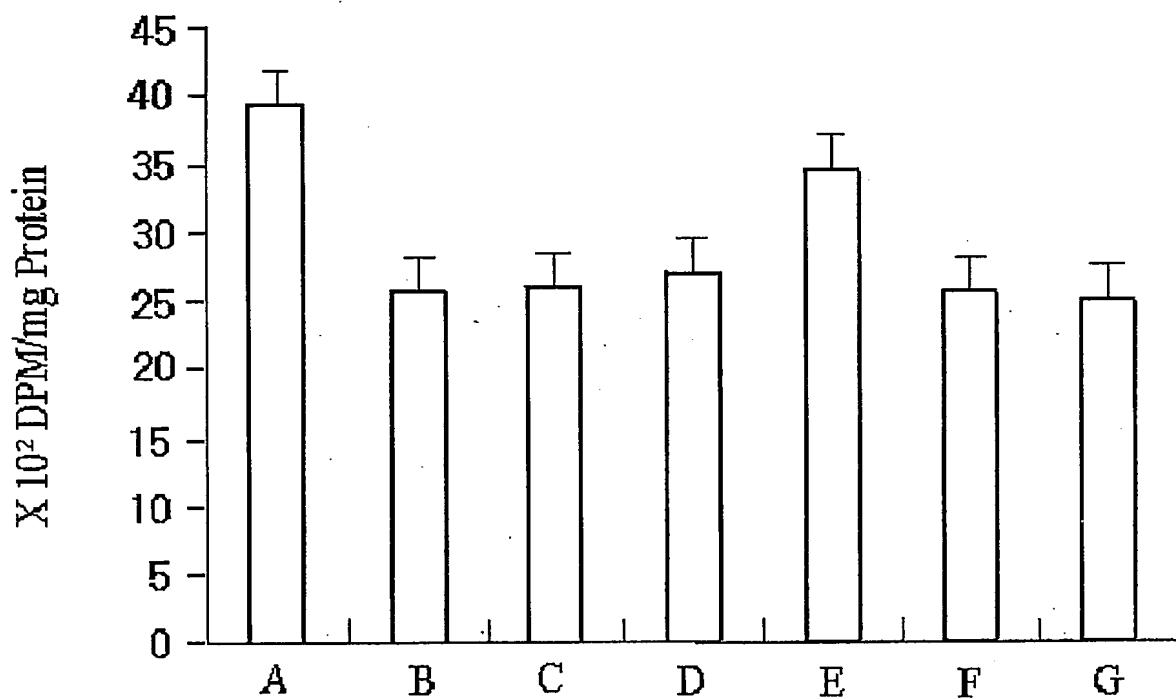
1: Control

2: Bromobenzene 1mM

3: Bromobenzene 1mM + Methanol Insoluble Fr. 200 μ g/ml4: Bromobenzene 1mM + Methanol Soluble Fr. 200 μ g/ml

18/20

FIG. 14



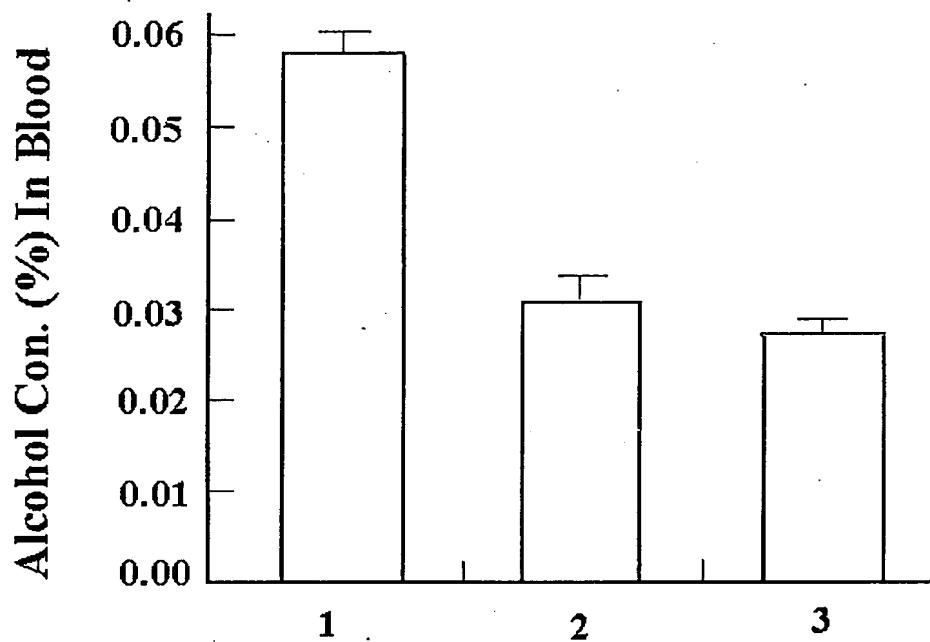
A: Control

B: Bromobenzene 1mM

C: Bromobenzene 1mM + Fr. 1 200 μ g/mlD: Bromobenzene 1mM + Fr. 2 200 μ g/mlE: Bromobenzene 1mM + Fr. 3 200 μ g/mlF: Bromobenzene 1mM + Fr. 4 200 μ g/mlG: Bromobenzene 1mM + Fr. 5 200 μ g/ml

19/20

FIG. 15



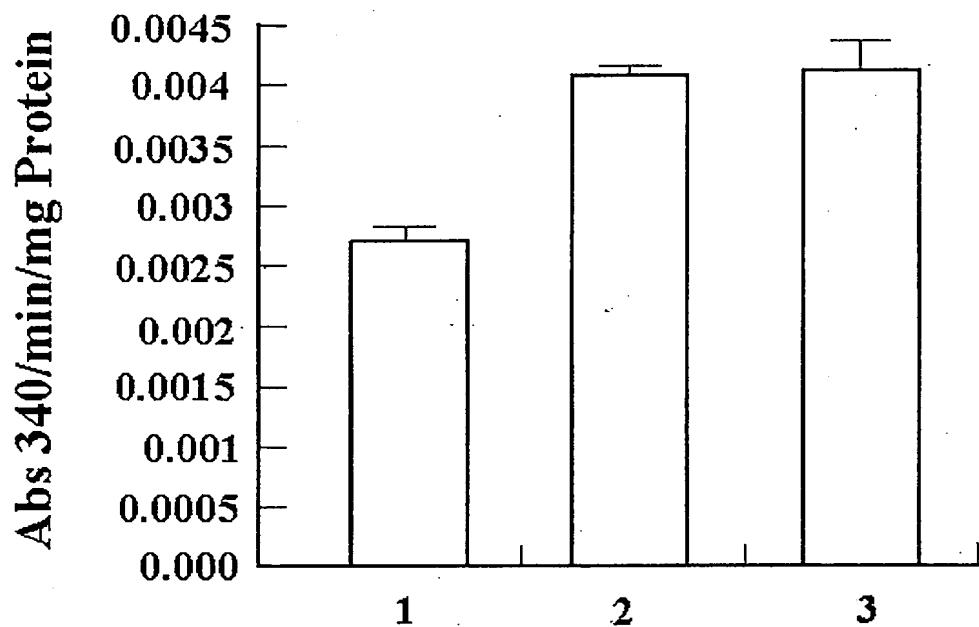
1: 40% Alcohol 2mℓ + Water 2mℓ

2: 40% Alcohol 2mℓ + 500mg/mℓ Insoluble Fr. 2mℓ

3: 40% Alcohol 2mℓ + 500mg/mℓ Polysaccharide Frcation 3 2mℓ

20/20

FIG. 16



1: 40% Alcohol 2mℓ + Water 2mℓ

2: 40% Alcohol 2mℓ + 500mg/ml Insoluble Fr. 2mℓ

3: 40% Alcohol 2mℓ + 500mg/ml Polysaccharide Fraction 3 2mℓ

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.